

IN THE SPECIFICATION:

Please insert the following unnumbered paragraph between paragraph [0001] and paragraph [0002]:

SEQUENCE LISTING

The instant application contains a Sequence Listing submitted pursuant to 37 C.F.R. § 1.52(e) via triplicate CD-R in lieu of a printed paper copy, and is hereby incorporated by reference in its entirety. Said CD-R, recorded on December 29, 2003, are labeled "CRF", "Copy 1", and "Copy 2", and each identically contains a single 950 Kb file (NAPRO18U.APP).

Please replace paragraph [0103] with the following amended paragraph:

[0103] Figure 1. *Diagram of pAURHYG(x)eGFP target plasmids.* Sequences are shown for the **normal wild type** hygromycin resistance allele (SEQ ID NO: **4341**) and the mutant alleles present in pAURHYG(rep)eGFP (SEQ ID NO: **4342**), pAURHYG(ins)eGFP (SEQ ID NO: **4343**) and pAURHYG(Δ)eGFP (SEQ ID NO: **4344**). **The amino acid translation of the wt allele is shown in SEQ ID NO: 4345, while the common amino acid translation of the mutant alleles is shown in SEQ ID NO: 4346.**

Please replace paragraph [0104] with the following amended paragraph:

[0104] FIG. 2. *Dual targeting protocol.* (A) Schematic diagram of the generalized strategy for dual targeting. (B) Sequences of the hygromycin-

resistance gene and its ~~mutation~~ mutations (SEQ ID NOS 4341, 4342, 4347 respectively for the wt, the mutant -- i.e., replacement --, and the converted alleles) and of the Hyg3S/74NT sequence-altering oligo (SEQ ID NO: 4348).

(C) Schematic of the YAC containing the human β -globin locus (SEQ ID NO: 4349) and the β Thal1 (SEQ ID NO: 4350) and β Thal2 (SEQ ID NO: 4351) sequences that are changed by the corresponding oligonucleotides.

Please replace paragraph [0105] with the following amended paragraph:

[0105] FIG. 3. *Dual targeting results.* (A) Efficiency of gene editing of hygromycin mutation using the dual targeting protocol. For these experiments, YAC-containing LSY678IntHyg(rep) β cells are grown in the presence of HU, electroporated with the selectable and nonselectable oligonucleotides, and allowed to recover in the presence of TSA. (B) Gene editing of the human β -globin gene directed by the β Thal1 oligonucleotide, including the sequence of the altered segment before (SEQ ID NO: 4352) and after (SEQ ID NO: 4353) the conversion. The 13-mer sequence in the bottom-most panel is shown in SEQ ID NO: 4354.

Please replace paragraph [0106] with the following amended paragraph:

[0106] FIG. 4. *Dual targeting and Rad51.* (A) Efficiency of gene editing of hygromycin mutation using the dual targeting protocol in combination with overexpression of yeast Rad51. For these experiments, YAC-containing LSY678IntHyg(rep) β cells are grown in the presence of HU, electroporated with the selectable and nonselectable oligonucleotides, and allowed to recover in the presence

of TSA. (B) Gene editing of the human β -globin gene directed by the β Thal2 oligonucleotide, including the sequence of the altered segment before (SEQ ID NO: 4355) and after (SEQ ID NO: 4356) the conversion. The 14-mer sequence in the bottom-most panel is shown in SEQ ID NO: 4357.

Please replace paragraph [0178] with the following amended paragraph:

[0181] We also use additional oligonucleotides to assay the ability of individual oligonucleotides to correct multiple mutations in both the pAURHYG(x)eGFP plasmid and in yeast strains with integrated copies of pAUR101-HYG(x)eGFP. These include, for example, an oligonucleotide that alters two basepairs that are 3 nucleotides apart with the sequence 5'-CTC GTG CTT TCA GCT TCG ATG TAG GAG GGC GTG GGT **ACG** TCC TGC GGG TAA ATA GCT GCG CCG ATG GTT TCT AC-3' (SEQ ID NO: 4358); a 74-mer that alters two basepairs that are 15 nucleotides apart with the sequence 5'-CTC GTG CTT TCA GCT TCG ATG TAG GAG GGC GTG GAT **ACG** TCC TGC GGG TAA **ACA** GCT GCG CCG ATG GTT TCT AC-3' (SEQ ID NO: 4359); and a 74-mer that alters two basepairs that are 27 nucleotides apart with the sequence 5'-CTC GTG CTT TCA GCT TCG ATG TAG GAG GGC GTG GAT **ACG** TCC TGC GGG TAA ATA GCT GCG CCG **ACG** GTT TCT AC (SEQ ID NO: 4360). The nucleotides in these oligonucleotides that direct alteration of the target sequence are in boldface. These oligonucleotides are chemically modified to enable them to effect oligonucleotide-directed nucleic acid sequence alteration.

Please replace Table 1, located immediately below paragraph [0178], with the following amended Table 1:

Table 1: Oligonucleotides used in Examples 1 and 2

Name	Size	Sequence	SEQ ID NO:
Hyg3S/74T	74mer	5'-C*T*C* GTG CTT TCA GCT TCG ATG TAG GAG GGC GTG GAT ACG TCC TGC GGG TAA ATA GCT GCG CCG ATG GTT TC *T*A*C-3'	<u>4361</u>
Hyg3S/74NT	74mer	5'-G*T*A* GAA ACC ATC GGC GCA GCT ATT TAC CCG CAG GAC GTA TCC ACG CCC TCC TAC ATC GAA GCT GAA AGC AC*G *A*G-3'	<u>4362</u>

Phosphorothioate linkages are indicated as "*" between the bases. The base corresponding to the location of the replacement mutation in the Hyg(rep) target is in bold.

Please replace paragraph [0179] with the following amended paragraph:

[0179] In this example we demonstrate that the efficiency of alteration of nucleic acid sequence at a second site is enhanced in a population of nucleic acid molecules that have been previously selected for alteration at a first target site. We use yeast strains having an integrated copy of the pAUR101-HYG(rep)eGFP plasmid described in Example 1 that also contain the β S YAC (230 kb YAC comprising the human β -globin gene). In some experiments, the yeast strains also contain a plasmid that overexpresses yeast Rad51, designated pYNARad51. We use several oligonucleotides: Hyg3S/74NT, which, as described in Example 1, is capable of directing alteration of the mutated Hyg(x)eGFP target to confer hygromycin resistance; and β S-386m and β S-378m, each of which is a 71-mer oligonucleotide with 3 phosphorothioate linkages on each end, which is capable of directing a mutation in the human β -globin gene. The sequence of β S-386m is 5' – G*C*C* TCA CCA CCA ACT TCA TCC ACG TTC ACC TTG CCT CAC AGG GCA GTA ACG GCA GAC TTC TCC ACA GG*A *G*T – 3' (SEQ ID NO: 4350) and the sequence of β S-378m is 5' – T*A*A* CGG CAG ACT TCT CCA CAG GAG TCA GGT GCA CCG TGG

TGT CTG TTT GAG GTT GCT AGT GAA CAC AG*T *T*G – 3' (SEQ ID NO: **4351**).

βS-386m and βS-378m both hybridize to the non-transcribed sequence of the human β-globin gene and direct a nucleic acid sequence alteration that creates a β-thalassemia mutation: βS-386m converts a TGG codon to a stop codon (TGA) and βS-378m converts the ATG start codon to ACG.

Please replace paragraph [0180] with the following amended paragraph:

[0180] We introduce the oligonucleotides into the yeast cells by electroporation as follows: we prepare electrocompetent yeast cells by inoculating a single colony into 10 ml of appropriate media supplemented with 250 µg/ml aureobasidin and grow the cultures overnight with shaking at 300 rpm at 30°C. In this Example, we use YPD media unless the cells contain a plasmid to overexpress a repair protein when we use SC-ade media. We dilute the cells into 40 ml media to an initial OD₆₀₀ of approximately 0.15. We incubate the cells with shaking at 30°C until the OD₆₀₀ is approximately 0.25 and, in some experiments, we add 100 mM hydroxyurea (HU). We continue to incubate the cells with shaking at 30°C until the OD₆₀₀ is approximately 0.6. We spin down the cells at 3000 rpm for 5 minutes and resuspend in 1 ml YPD supplemented with 25 µl 1M DTT and incubate the culture with shaking at 30°C for 20 minutes. We wash the cells four times by centrifuging at 4°C at 3000 rpm for 5 minutes and resuspending the cells twice in 25 ml ice-cold distilled water; once in 25 ml ice-cold 1M sorbitol; and once in 1 ml ice-cold 1M sorbitol. We centrifuge the cells at 4°C at 5000 rpm for 5 minutes and resuspend the cells in 120 µl 1M sorbitol. We transform the electrocompetent cells with Hyg3S/74NT and either βS-386m or βS-378m by mixing 40 µl of cells with 30 µg oligonucleotide (or 30 µg of each oligonucleotide) and incubate on ice for 5 minutes. We transfer the mixture to a 0.2 cm electroporation cuvette and electroporate with a BIO-RAD® Gene

Pulser® apparatus at 1.5 kV, 25 µF, 200 Ω for one approximately five-second pulse. We then resuspend the cells in 3 ml YPD media supplemented with Aureobasidin and allow the cells to recover by incubating them overnight at 30°C with shaking at 300 rpm. In some experiments, we supplement the culture with 50 mg/ml Trichostatin A (TSA) during this recovery phase. We spin down the cells, resuspend in 1 ml YPD and spread 100 µl of this culture (and/or 100 µl of 10¹ or 10² dilutions) on selective plates containing 300 µg/ml hygromycin and spread 200 µl of a 10⁵ dilution of this culture on selective plates containing 500 ng/ml aureobasidinA and incubate at 30°C for 3 days to allow individual yeast colonies to grow. We count the colonies on the plates and calculate the conversion efficiency for the hygromycin target by determining the number of hygromycin resistance colonies per 10⁵ aureobasidinA resistant colonies. We pick individual colonies from YPD-hygromycin plates into 96-well plates with 150 µl YPD/well and allow the cells to grow overnight at 30°C. We screen individual wells for alteration of the β-globin target in the βS YAC as follows: we PCR amplify a 345 bp fragment of the β-globin gene using forward primer PCO2; 5'- TCC TAA GCC AGT GCC AGA AGA -3' (SEQ ID NO: 4375) and reverse primer PCO5; 5'- CTA TTG GTC TCC TTA AAC CTG -3' (SEQ ID NO: 4363) and purify the PCR product. We analyze the sequence at the target nucleotide in the PCR products by SNaPshot™ analysis on an ABI3100 (Applied Biosystems™) using primers corresponding to the different alterations directed by βS-386m and βS-378m as follows: 386RC; 5'- CCC CCC CCC CCC CCC CCA AGT CTG CCG TTA CTG CCC TGT G -3' (SEQ ID NO: 4364) and 378MF; 5'- TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TGC AAC CTC AAA CAG ACA CCA -3' (SEQ ID NO: 4365).

Please replace paragraph [0183] with the following amended paragraph:

[0183] *Assay system.* We monitor targeted alteration of genetic material in human blood cells using the chromosomal gene encoding the beta subunit of hemoglobin as the target. We cointroduce two oligonucleotides with a plasmid comprising a mutant copy of the green fluorescent protein (GFP) gene. The second oligonucleotide is designed to direct an alteration which repairs the mutant GFP resulting in fluorescence. The first oligonucleotide is designed to convert the wild-type allele to the sickle allele. We use first oligonucleotides that correspond in sequence to the wild-type allele at all positions except the single nucleotide position designed to introduce the sickle mutation into the gene. Therefore, these oligonucleotides are identical to the oligonucleotides described in Example 6 and shown in Table 7 except for a single base. For example, we use first oligonucleotides selected from: 5'-C*A*A* CCT CAA ACA GAC ACC ATG GTG CAC CTG ACT CCT GtG GAG AAG TCT GCC GTT ACT GCC CTG TGG GGC AA*G *G*T -3'; SEQ ID NO: **4366**; 5'-A*C*C* TTG CCC CAC AGG GCA GTA ACG GCA GAC TTC TCC aCA GGA GTC AGG TGC ACC ATG GTG TCT GTT TGA GG*T *T*G-3'; SEQ ID NO: **4367**; 5'-ACC TCA AAC AGA CAC CAT GGT GCA CCT GAC TCC TGt GGA GAA GTC TGC CGT TAC TGC CCT GTG GGG CAA GG -3'; SEQ ID NO: **4368**; 5'-G*A*C* ACC ATG GTG CAC CTG ACT CCT GtG GAG AAG TCT GCC GTT ACT GCC *C*T*G -3'; SEQ ID NO: **4369**; and 5'-A*C*C* TCA AAC AGA CAC CAT GGT GCA CCT GAC TCC TGt GGA GAA GTC TGC CGT TAC TGC CCT GTG GGG CA*A *G*G -3' (**SEQ ID NO: 4370**). The bases in the oligonucleotides which are mismatched to the wild-type allele are shown in lowercase. The oligonucleotides are synthesized with three phosphorothioate linkages on each end (represented with asterisks) or with a single LNA base at each end (bold).

Please replace paragraph [0185] with the following amended paragraph:

[0185] We electroporate the oligonucleotides and the GFP plasmid into the cells under square wave conditions as follows. We add 250 μ l cell suspension, 5 μ g GFP plasmid and 30 μ g each oligonucleotide to a 2 mm gap cuvette and electroporate for one 19 msec pulse at 220 V. We then add 750 μ l Iscove's Medium (Invitrogen™), 10% FCS (StemCell Technologies) and the cytokines flt-3, SCF, TPO at 100 ng/ml final concentration, glutamine and penicillin/streptomycin. Alternatively, we add 250 μ l cell suspension, 250 μ l QBSF-60 medium supplemented with flt-3, SCF and TPO and 30 μ g oligonucleotide to a 4 mm gap cuvette and electroporate for five 19 msec pulse at 220 V with a pulse interval of 1 sec. We then add 500 μ l Iscove's Medium (Invitrogen™), 10% FCS (StemCell Technologies) and the cytokines flt-3, SCF and TPO at 100 ng/ml final concentration. We select for the repair of the mutant GFP protein using by FACS and analyze the sequence of the hemoglobin target by PCR amplification and analysis on the SNaPshot™ device using two oligonucleotides: 5'- TTT TTT TTT TTT TTT GAC ACC ATG GTG CAC CTG ACT CCT G -3'; SEQ ID NO 4371; and 5'- TTT TTT TTT TTT TTT TTT TTC AGT AAC GGC AGA CTT CTC C -3'; SEQ ID NO 4372. As we see in other cells, these oligonucleotides direct targeted alteration in human blood cells.

Please replace paragraph [0187] and with the following amended paragraph:

[0187] The dual targeting strategy is illustrated in FIG. 2A. The LSY678IntHyg(rep) β strain (Table 5) contains a 240 kb human β^S -globin YAC and a cassette containing a chromosomal hygromycin-resistance gene inactivated by a single base mutation and a functional aureobasidin-resistance gene. See Liu et al., *Nucleic Acids Res.* 31:2742-2750 (2002); Parekh-Olmedo et al., *Chem. Biol.* 9:1073-1084 (2002); and Liu et al., *Mol. Cell Biol.* 22:3852-3863 (2002). FIG. 2B shows the oligonucleotide that is used to direct editing of the chromosomal hygromycin mutant gene. Hyg3S/74NT (SEQ ID NO: 4348) is a 74-mer that is specific for binding to the nontranscribed strand and contains three terminal phosphorothioate

linkages. *Id.* Also shown is the target sequence of the mutant, which contains a TAG stop codon. FIG. 2C illustrates the structure of the β -globin YAC and nucleotides targeted for editing are specified. The two nonselectable changes are directed by different oligonucleotides, β Thal1 (SEQ ID NO: 4350) and β Thal2 (SEQ ID NO: 4351), in separate experiments. The YAC contains approximately 230 kb of genomic DNA from human chromosome 11, indicated by the shaded region. The unshaded regions represent the yeast sequences that are on either end of the YAC (not drawn to scale). Yu et al., *Proc. Natl. Acad. Sci. USA* 97:5978-5983 (2000). A portion of the β -globin sequence is shown, beginning with the start codon. β Thal1 directs a change from a G to an A while β Thal2 directs a change from a T to a C. The sequences of the oligonucleotides having nucleic acid sequence alteration activity are shown and are designed to bind to the non-transcribed strand, relative to human transcription of the β -globin locus. Both changes result in single-base substitutions that have been documented to result in β -thalassemia in humans.

Please replace paragraph [0194] with the following amended paragraph:

[0194] *Oligonucleotides.* Hyg3S/74NT (SEQ ID NO: 4348), β Thal1 (SEQ ID NO: 4350), and β Thal2 (SEQ ID NO: 4351) are ordered from IDT with HPLC purification. Hyg3S/74NT is a 74mer and both β Thal1 and β Thal2 are 71mers; all three oligonucleotides have three phosphorothioate linkages at the 5' and 3' ends (FIG. 2).

Please replace paragraph [0196] with the following replacement paragraph:

[0196] Individual colonies are picked from the hygromycin agar plates into 96-well plates (Corning) containing 150 μ l YPD and grown overnight at 30°C with shaking. A 345 bp PCR product specific for the human β -globin locus is amplified from each of the 96 wells using the primers PCO2 (5'-TCCTAAGCCAGTGCCAGAAG-3' (SEQ ID NO.: 4373)) and PCO5 (5'-

CTATTGGTCTCCTTAAACCTG-3' (SEQ ID NO.: 4374) in order to screen for the β Thal1 or β Thal2 conversion. The PCR reactions are performed by adding 8 pmoles of each primer and 2.5 μ l yeast cell culture into pre-aliquoted PCR reaction mixes (Marsh/Abgene). The PCR reactions use an annealing temperature of 45.8°C and an extension time of 1 min for 35 cycles. The PCR reactions are purified using a QiaQuick PCR 96-well purification kit (Qiagen) and eluted in a volume of 80 μ l. One microliter of the purified PCR product is used as a template for the ABI SNaPshot reaction. The sequence of the SNaPshot primer used to screen for the β Thal1 conversion is: 5'-CCCCCCCCCCCCCCCCCAAGTCTGCCGTTACTGCCCTGTG-3' (SEQ ID NO: 4364). The sequence of the SNaPshot primer used to screen for the β Thal2 conversion is: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCACAGGAGTCAGGTGCACC-3' (SEQ ID NO: 4365). The SNaPshot reactions are performed using an ABI Prism SNaPshot Multiplex Kit, as specified by the manufacturer, and analyzed on an ABI 3100 Genetic Analyzer.